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## Preservation of hippocampal neuron numbers in aged rhesus monkeys

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### Abstract

To investigate whether or not aging of nonhuman primates is accompanied by a region-specific neuron loss in the hippocampal formation, we used the optical fractionator technique to obtain stereological estimates of unilateral neuron numbers of the hippocampi of eight young (0–4 years) and five aged (18–31 years) male rhesus monkeys (*Macaca mulatta*). Our results show a preservation of neurons (mean  $\times 10^3 \pm \text{S.D.} \times 10^3$ ) in the subiculum (young =  $588 \pm 124$ , aged =  $612 \pm 207$ ), CA1 (young =  $1051 \pm 249$ , aged =  $1318 \pm 311$ ), CA2 (young =  $100 \pm 18$ , aged =  $113 \pm 12$ ), CA3 (young =  $478 \pm 125$ , aged =  $509 \pm 139$ ), hilus (young =  $337 \pm 115$ , aged =  $394 \pm 90$ ), and dentate gyrus (young =  $5550 \pm 1725$ , aged =  $7799 \pm 2087$ ) of the hippocampal formation. These results confirm a previous stereological study in rhesus monkeys, but are in conflict with data for humans, showing age-dependent region-specific alterations in the hippocampal formation.

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**Keywords:** Aging; Rhesus monkey; Hippocampal formation; Neuron number; Stereology; Optical fractionator

### 1. Introduction

Numerous efforts have been made to answer the question whether neuronal loss in the hippocampal formation and entorhinal cortex can, at least in part, accounts for age-related decline in cognitive processes such as learning and memory [4,5,21,37,38]. In earlier studies, most of the data were reported as neuron density per unit volume or area, but this literature on neuronal numbers in the hippocampal formation, for example, shows much controversy. Geinisman et al. [15] extensively reviewed the data on neuronal numbers and densities in the hippocampus of aging subjects. Whereas some studies reported no age-associated loss in human hippocampal fields CA1–4 and the subiculum [10], other studies demonstrated a decrease of neuron numbers or densities in all or some of the hippocampal subfields or the dentate gyrus with advancing age [7,32,36,39]. Most, but not all, of these earlier studies made assumptions about size, shape, and distribution of the neurons, and for that reason these techniques are now designated as assumption-based counting techniques. The commonly used correction factor of Abercrombie is based on the assumption that neurons are

spheres of constant diameter [28]. In addition, other corrections could compensate for hippocampal or hemisphere volume and/or fixation-induced postmortem tissue shrinkage. As long as the biological material meets the assumptions of the corrections, the number estimates are not biased. However, in most cases, it is more likely that the assumptions are not valid [28].

More recent quantitative studies on neuronal numbers have one element in common: they aim at obtaining estimates of the total number of neurons, e.g. in the hippocampal formation, with design-based methods [23,61,62,64,67,68]. Since 1984, when Sterio first described the disector [56], the use of assumption-free, unbiased counting methods was gradually introduced into the study of age-related changes in neuronal numbers. Design-based methods, collectively called stereology, have the valuable characteristic that no assumptions about the size, shape, and distribution of the neurons need to be made. The only requirement for such a study is the accessibility of the complete, intact brain structure of interest. Because of the mathematical approach, there is a possibility to optimize the counting procedure during a pilot study, and the total group variance can be split into the biological variance and the variance contribution from the method, e.g. when comparing two experimental groups [23,26,62,67,68].

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The ultimate goal in neurobiological research on aging is to understand the mechanisms taking place in human subjects, e.g. that eventually lead to a decreased performance in hippocampus-dependent memory. A number of reports used stereological techniques to assess hippocampal pyramidal neuronal estimates in human aging. West [63] investigated the hippocampal formations of 32 male human subjects (13–85 years), and reported a region-specific loss in the hilus and subiculum. Simic et al. [55] performed a similar quantitative analysis with the hippocampal formations of 18 human subjects (16–99 years). In agreement with the former study [63], they found an age-associated decrease in the estimated neuronal number in the subiculum. However, the latter study was not able to confirm the loss of hilar neurons, as had been found by West [63]. In contrast, Simic et al. [55] reported a loss of CA1 pyramidal neurons with advancing age, whereas West [63] did not.

To better understand these differential aging-related effects, it is necessary to investigate the underlying mechanisms, preferentially in appropriate animal models. A requirement then is that the particular species has to develop similar age-related changes in hippocampal neuron numbers. Rodents are often used for aging studies, because they age relatively fast, and are easy to have access to. Interestingly, two stereological studies in behaviorally characterized rats failed to demonstrate an age-associated neuron loss in the hippocampal formation [50,51].

The rhesus monkey (*Macaca mulatta*) may provide a good animal model, since it is a species close to humans in which a full range of neurobiological studies can be conducted [53]. Rhesus monkeys are considered to serve as a useful model for normal human aging [58] because aged nonhuman primates show many of the age-related behavioral [4,21,37,38] and neurobiological alterations, such as changes in neurons, dendrites and synapses, and in neurotransmitter and brain metabolite concentrations [6,20,37,58,59], that also have been reported in aging humans. Rhesus monkeys in laboratory conditions can reach the age of 35–40 years and may be considered as old when they are between 20 and 25 years. Rhesus monkeys over 28 years of age are commonly regarded as the oldest of the old [38,58].

Data on the impact of aging on hippocampal neuron numbers in rhesus monkeys, however, is scarce. An assumption-based study reported an age-related neuron loss in the CA1 region [9], whereas another nonstereological study showed no significant difference in the CA1 region or the subiculum [52]. However, one report that used a stereological approach showed no decrease in CA1 neuronal number, but a decreasing trend in the subiculum [65].

The aim of the present study was to obtain stereological data of a larger number of male rhesus monkeys to further investigate whether regionally specific neuron loss occurs in the nonhuman primate hippocampal formation similar as in aging humans, or whether aging is not associated with a significant hippocampal neuron loss. By circumventing

confounding factors, such as differential environmental influences and high genetic variation, rhesus monkeys of different ages from a single colony may provide a good model approach to investigate age-related changes in neuronal number in more detail.

## 2. Methods

### 2.1. Tissue preparation

Brain tissue blocks including the entire hippocampal formation were obtained from autopsies of 13 male rhesus monkeys (*M. mulatta*) from the Wisconsin Regional Primate Research Center (Madison, USA), and stored in buffered formalin. The brain of one aged monkey was perfused with 4% paraformaldehyde in phosphate buffer (pH 7.4). In the present study, we used hippocampi of eight young (0–4 years) and five aged (18–31 years) animals. None of the animals suffered from neurological diseases. Postmortem intervals between death and autopsy varied between 30 min and 15 h.

Using a multiblade tissue slicer with variable spacers [44], the hippocampi were cut into 3.2 mm slabs. This macroscopical cutting protocol allows sampling slabs in a multi-stage fractionator design [24,62]. The cutting edges of the parallel knives were kept perpendicular to the longitudinal hippocampal axis at the level of the main body, shortly behind the uncus part of the hippocampus. The position of the knives to the hippocampus along its axis was random. It was decided randomly for each animal whether the even or the odd slabs, containing hippocampal tissue, were embedded in glycolmethacrylate resin (Technovit 7100; Kulzer, Weinheim, Germany; see also [26]). In short, the selected slabs were washed in double distilled water for 24 h, dehydrated through 70 and 96% ethanol (8 and 16 h, respectively), pre-infiltrated with 96% ethanol and base liquid Technovit 7100 (1:1, v/v) for 8 h, and infiltrated with pure base liquid Technovit 7100 with hardener I for 48 h. Then, the tissue slabs were polymerized in Technovit 7100 containing hardener I and II in plastic molding cup trays at 4 °C. After 2 days, the polymerized tissue blocks were mounted with Technovit 3400 (Kulzer).

All polymerized tissue slabs were serially sectioned at 30 µm thickness using a Leica RM 2065 rotating microtome and a histoknife with D-grinding on a 5° cutting angle. All sections that contained hippocampal tissue were collected in series on glass slides while floating on a water bath (room temperature), and immediately dried on a hot plate of 60 °C. Sections were additionally dried overnight in an oven at the same temperature.

Staining of the sections was performed at room temperature with a mixture of Cresyl violet, Methylene blue, and Azur II. After drying on a hot plate of 60 °C, the sections were mounted with Eukitt and coverslipped. All procedures have been described in detail previously [26].

## 2.2. Stereological evaluation

To estimate the total unilateral neuron number in all subfields of the rhesus monkey hippocampal formation, the optical fractionator technique with a multistage fractionator sampling design was applied with Stereoinvestigator 3.16 software (MicroBrightField, Colchester, VT, USA). All measurements were carried out with a Zeiss III RS microscope (Carl Zeiss, Oberkochen, Germany), to which a defined stepping motor in  $x,y$ -axes (LUDL Electronic Products, Hawthorne, NY, USA) and an electronic microcator (Heidenhahn MT 12, Dr. Joh. Heidenhahn GmbH, Traunreut, Germany) were attached (for details on setup, see [26]). All experimental parameters for the rhesus monkey hippocampal formation were established in a pilot experiment. Every 17th section was selected (Table 1), of which the first was chosen with a random number. Because the neurons of the subiculum, Ammon's horn, and hilus are rather disperse, the evaluations in these subfields were performed with a 40 $\times$  objective lens (N.A. 0.75). Although principal neurons are easily distinguished from glial cells by their size and the characteristic of a clear, large, and mostly centrally situated nucleus, we might unintentionally have included in our evaluations a small percentage ( $\pm 2\%$ ; Czeh and Seress, personal communication) of interneurons in the neuron-containing layers of the subiculum and Ammon's horn. The granule cells of the dentate gyrus are densely packed, and could be evaluated using a 100 $\times$  oil objective lens (N.A. 1.3). The area and the height of the optical disector,  $a(\text{frame})$  and  $h$ , respectively, and the area of the  $x$  and  $y$  steps,  $A(x,y \text{ step})$ , for each subfield are listed in Table 1. The section thickness was measured during the stereological counting procedure at five random places within each selected section with a 100 $\times$  oil objective lens (N.A. 1.3). With these settings, 1–2 neurons were counted per frame in each subfield on average. Prior to the counting procedure, the animal numbers were coded, and the code was only revealed when all data had been collected. Individual estimates of the total neuronal number ( $N$ ) for each subfield of the hippocampal formation were calculated according to the following formula:

$N = \sum Q^- \times 1/\text{slab sf} \times 1/\text{ssf} \times 1/\text{asf} \times 1/\text{tsf}$ , with  $\sum Q^-$  as the sum of counted neurons per subfield per animal, slab sf = slab sampling fraction, ssf = section sampling fraction, asf = area sampling fraction, and tsf = thickness sampling fraction (Table 1). The corresponding individual CE (coefficient of error) were calculated with the formula as described in [26]. This is a modified formula for systematic samples from a continuous brain structure with a relatively high homogeneity in neuron distribution [19], thus for systematically sampled sections along the long axis of the primate hippocampal formation.

The group means of the neuronal estimates of the young and aged rhesus monkeys were tested per subfield with the two-tailed unpaired Student's  $t$ -test, and the significance level was defined at  $\alpha = 0.05$ .

## 2.3. Delineation of the hippocampal subfields

All delineations were made by using a low-power magnification. First, the complete section was observed through the microscope, before the delineation on the computer screen was done. The borders of the neuron-containing subfields were defined according to criteria from the human [1] and rhesus monkey [53] hippocampal formation, and from a study of Lorente de N6 [29]. Delineations at various longitudinal levels through the hippocampal formation are shown in Fig. 1 from rostral to caudal. Since the sections were made perpendicular to the hippocampal formation axis at the most proximal main body, and not coronally, it was not possible to appoint coordinates of a rhesus monkey brain atlas to the sections. Fig. 1A–C shows sections through the uncus part of the hippocampal formation, the proximal main body is shown in Fig. 1D, and the section in Fig. 1E is taken from the posterior main body.

The border between the subiculum, also called the subiculum proper, and the CA1 subfield is oblique, and often characterized by a cell-free zone. The point where the stratum radiatum of the CA1 is vanishing, represents one end of the oblique border. The other end starts where the subiculum pyramidal layer contains a variety of smaller neurons within

Table 1

Experimental parameters used with the optical fractionator: amount of counted neurons,  $\sum Q^-$ ; area of the optical disector counting frame,  $a(\text{frame})$ ;  $x$  and  $y$  step sizes,  $A(x,y \text{ step})$ ; area sampling fraction, asf ( $=a(\text{frame})/A(x,y \text{ step})$ ); section thickness,  $t$ ; height of optical disector,  $h$ ; thickness sampling fraction, tsf ( $=h/t$ ); section sampling fraction, ssf; slab sampling fraction, slab sf

	Mean $\sum Q^- \pm \text{S.D.}$	$a(\text{frame})$ ( $\mu\text{m}^2$ )	$A(x,y \text{ step})$ ( $\mu\text{m}^2$ )	asf	Mean $t \pm$ S.D. ( $\mu\text{m}$ ) <sup>c</sup>	$h$ ( $\mu\text{m}$ )	tsf	Mean number of sections $\pm$ S.D.	ssf	slab sf
Subiculum <sup>a</sup>	429 $\pm$ 117	75 $\times$ 60	350 $\times$ 350	0.0367	19.65 $\pm$ 0.93	15	0.64–0.73	17 $\pm$ 3	1/17	1/2
CA1 <sup>a</sup>	383 $\pm$ 99	75 $\times$ 60	525 $\times$ 525	0.0163	19.65 $\pm$ 0.93	15	0.64–0.73	18 $\pm$ 2	1/17	1/2
CA2 <sup>a</sup>	272 $\pm$ 144	75 $\times$ 60	200 $\times$ 200	0.1125	19.65 $\pm$ 0.93	15	0.64–0.73	16 $\pm$ 3	1/17	1/2
CA3 <sup>a</sup>	373 $\pm$ 128	75 $\times$ 60	350 $\times$ 350	0.0367	19.65 $\pm$ 0.93	15	0.64–0.73	17 $\pm$ 3	1/17	1/2
Hilus <sup>a</sup>	265 $\pm$ 86	75 $\times$ 60	350 $\times$ 350	0.0367	19.65 $\pm$ 0.93	15	0.64–0.73	17 $\pm$ 3	1/17	1/2
Dentate gyrus <sup>b</sup>	367 $\pm$ 124	15 $\times$ 15	275 $\times$ 275	0.0030	19.65 $\pm$ 0.93	15	0.64–0.73	17 $\pm$ 3	1/17	1/2

<sup>a</sup> All evaluations were performed with a 40 $\times$  objective lens (N.A. 0.75).

<sup>b</sup> All evaluations were performed with a 100 $\times$  oil objective lens (N.A. 1.3).

<sup>c</sup> Section thickness was measured with a 100 $\times$  oil objective lens (N.A. 1.3).

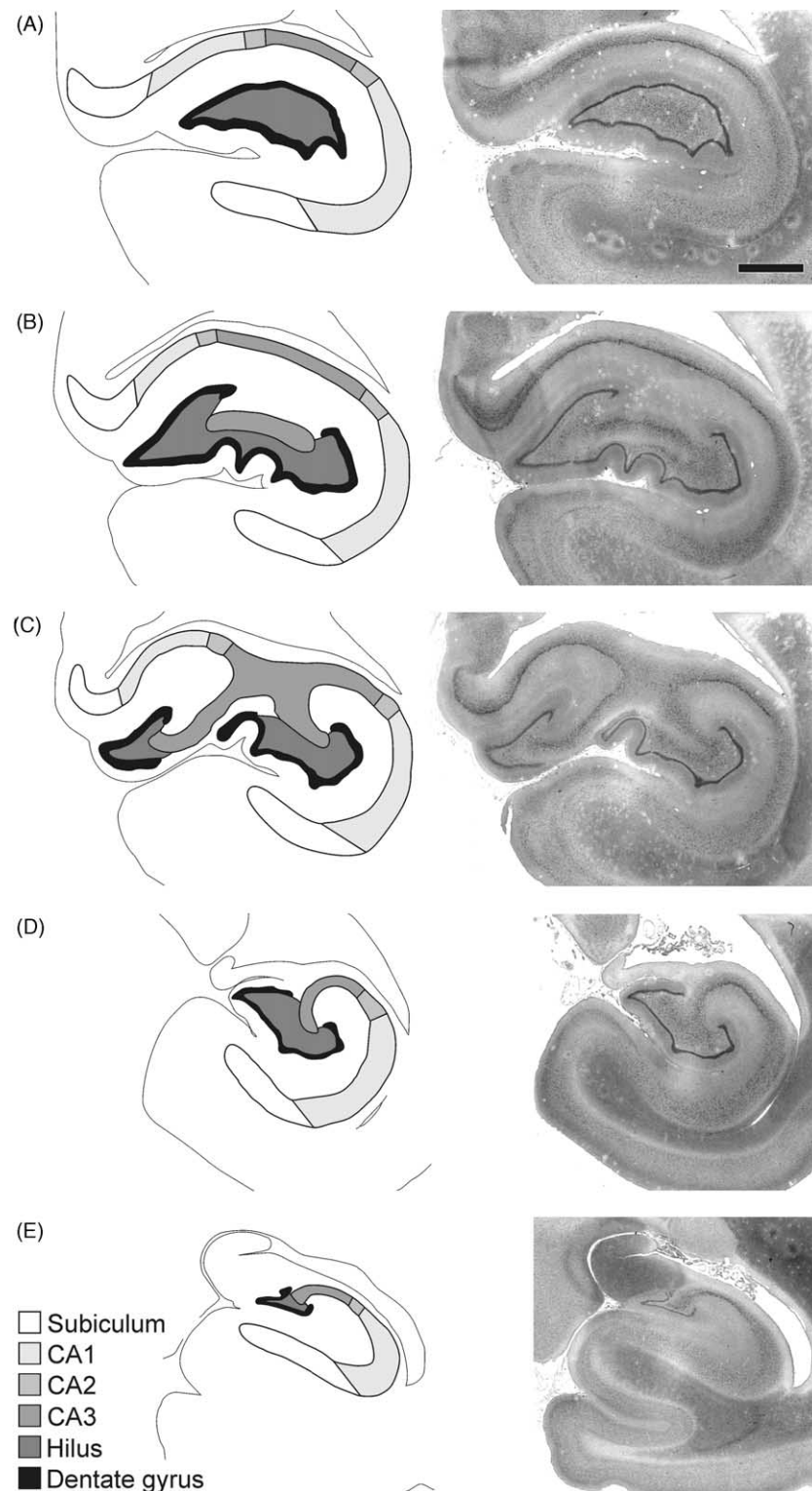


Fig. 1. Delineation of subfields subiculum, CA1, CA2, CA3, hilus, and dentate gyrus along the anterior-posterior axis of the rhesus monkey hippocampal formation. Sections were made perpendicular to the proximal main body (D). Sections through the uncus part of the hippocampal formation (A–C). Posterior main body (E). Calibration bar, for A–E, is 200  $\mu$ m.



the stratum oriens. The prosubiculum was included in the CA1 subfield.

The boundary between the CA1 and CA2 subfields was placed shortly after the pyramidal cell layer starts to broaden. CA2 neurons were more chromophilic than CA1 neurons, more densely packed, often contained more cytoplasm around the nucleus, and their dendrites occasionally bifurcated.

In contrast to previous studies, we defined the CA2 region as a separate subfield of the hippocampus, based on differential connectivity and vulnerability of this region in disease-related degenerative processes [1,70]. The border of the CA2 to the CA3 region was often characterized by a 'drop' of cells towards the hippocampal fissure, as was also observed for the CA1–CA2 border. Additionally, the course of the mossy fibers could be seen, of which the very ending of the bundle tapers into the CA2 [70].

The polymorphic layer of the dentate gyrus is formally called the hilus. However, in the present study, we additionally included those pyramidal neurons of the hippocampus that Lorente de N6 [29] originally designated as CA4. Because of the proximity of the cells in these subfields, it is difficult to precisely differentiate between these two functionally distinct regions [1]. However, to ensure constant and reproducible criteria, which is most important in stereological studies, we put together the polymorphic layer and the CA4 pyramidal neurons, and called this the hilus. This approach is identical to those used by West and Gundersen [67], West [63], and Leverenz et al. [27]. The pyramidal neurons of the hippocampus that were included in our present definition of the hilus, were those that made a sharp bend and folded back on themselves, i.e. those neurons that—compared to CA3 neurons—changed their orientation. The principal cells of the dentate gyrus are the granule cells that

form a separate cell layer of relatively small and densely packed neurons.

In the uncus of the hippocampal formation, eventual difficulties in delineating the subfields were overcome by following the borders of the subfields in sections along the hippocampal axis from the main body in rostral direction.

### 3. Results

Using the current experimental parameters for the optical fractionator that were established during a pilot experiment, the average number of counted neurons per subfield varied between 272 and 429 (Table 1), which is well beyond the recommendation of Gundersen and Jensen [18] to count 100–200 cells per structure per individual. Although Schmitz and Hof recommend to count between 700–1000 cells [54], we only slightly increased our cell counts compared to the recommendation of Gundersen and Jensen. This raise in counted neurons was sufficient in our study to compensate for an eventual higher method-derived variance because of the use of every second slab instead of the whole hippocampal formation. For example, the sectioning was not always in the ideal parallel plane of the slab surface. For this reason, some sections did not contain all or only incomplete hippocampal subfields. Counting particles in noncomplete regions does not affect the unbiased nature of the counting procedure, it merely increases the variance [43]. As a consequence of multistage sampling, the mean number of selected sections per hippocampal subfield varied between 16 and 18 (Table 1). The mean section thickness  $\pm$  S.D. was  $19.65 \pm 0.93$  as measured with a  $100\times$  oil lens (Table 1).

The individual estimated unilateral neuron numbers ( $N$ ) and the corresponding CEs in each hippocampal subfield

Table 2

Estimated individual unilateral neuron numbers ( $N$ ;  $\times 10^3$ ) with CE in the hippocampal subfields of young male rhesus monkeys: mean group numbers (mean  $N$ ), standard deviation (S.D.), and individual and mean CEs

Age	Subiculum		CA1		CA2		CA3		Hilus		Dentate gyrus	
	$N$	CE	$N$	CE	$N$	CE	$N$	CE	$N$	CE	$N$	CE
Newborn	657	0.053	1342	0.055	100	0.071	400	0.067	250	0.079	4817	0.064
Newborn	653	0.046	782	0.063	98	0.068	676	0.051	539	0.052	3833	0.066
1 day	650	0.048	1358	0.048	136	0.058	699	0.048	412	0.058	5503	0.057
13 days	347	0.066	880	0.061	74	0.081	387	0.068	162	0.094	3610	0.071
3.5 months	606	0.051	1085	0.057	109	0.068	256	0.075	300	0.072	4202	0.066
1 year	439	0.059	686	0.067	82	0.074	385	0.061	389	0.059	8111	0.046
4 years	458	0.058	1182	0.057	101	0.070	517	0.057	281	0.075	7243	0.052
4 years	654	0.049	1095	0.056	97	0.072	503	0.055	361	0.064	7078	0.050
Mean $N$ (CE)	558	0.054	1051	0.058	100	0.070	478	0.060	337	0.069	5550	0.059
S.D.	124		249		18		152		115		1725	
$CV^2 = (S.D./mean)^2$	0.0493		0.0561		0.0344		0.1016		0.1164		0.0966	
$CE^2$	0.0029		0.0034		0.0049		0.0036		0.0048		0.0035	
$BCV^2$	0.0464		0.0527		0.0295		0.0980		0.1116		0.0931	
$BCV^2$ (in % of $CV^2$ )	94		94		86		96		96		96	

$BCV^2 = CV^2 - CE^2$  (CE, coefficient of error; CV, coefficient of variation; BCV, biological coefficient of variation).

Table 3

Estimated individual unilateral neuron numbers ( $N$ ;  $\times 10^3$ ) with CE in the hippocampal subfields of aged male rhesus monkeys: mean group numbers (mean  $N$ ), standard deviation (S.D.), and individual and mean CEs

Age	Subiculum		CA1		CA2		CA3		Hilus		Dentate gyrus	
	$N$	CE	$N$	CE	$N$	CE	$N$	CE	$N$	CE	$N$	CE
18 years	486	0.054	1055	0.055	117	0.040	574	0.050	435	0.058	7706	0.060
21 years	597	0.052	1334	0.051	100	0.069	309	0.069	323	0.071	7850	0.054
24 years	962	0.040	1763	0.043	129	0.060	682	0.053	529	0.055	9693	0.045
29 years	583	0.052	1633	0.047	101	0.073	455	0.062	373	0.064	9333	0.046
31 years	431	0.059	1118	0.053	119	0.064	524	0.063	312	0.071	4413	0.064
Mean $N$ (CE)	612	0.052	1381	0.050	113	0.061	509	0.059	394	0.064	7799	0.054
S.D.	207		311		12		139		90		2087	
$CV^2 = (S.D./mean)^2$	0.1150		0.0508		0.0121		0.0746		0.0516		0.0716	
$CE^2$	0.0027		0.0025		0.0037		0.0035		0.0041		0.0029	
$BCV^2$	0.1123		0.0483		0.0084		0.0711		0.0475		0.0687	
$BCV^2$ (in % of $CV^2$ )	98		95		69		95		92		96	

$BCV^2 = CV^2 - CE^2$  (CE, coefficient of error; CV, coefficient of variation; BCV, biological coefficient of variation).

Table 4

Young vs. aged rhesus monkeys: mean estimated unilateral neuronal numbers ( $N$ ;  $\times 10^3$ ), and statistical results of the unpaired Student's  $t$ -test (two-tailed)

	Mean $N$		$P$ -value
	Young ( $n = 8$ )	Aged ( $n = 5$ )	
Subiculum	558	612	0.57
CA1	1051	1381	0.06
CA2	100	113	0.18
CA3	478	509	0.72
Hilus	337	394	0.36
Dentate gyrus	5550	7799	0.06

for young rhesus monkeys are shown in Table 2, as well as the mean group value of  $N$  with the standard deviation, and the mean CE. From these values, the biological variance  $BCV^2$  can be calculated according to the formula:  $CV^2 = CE^2 + BCV^2$  [17,67]. Table 3 shows the matching data for the aged rhesus monkeys. To make justified group comparisons, the  $BCV^2$  should contribute with more than 50% to the total observed variance  $CV^2$  [17,67]. In the current analysis, the observed group variance derived from interindividual differences, rather than from insufficient sampling (Tables 2 and 3). The unpaired Student's  $t$ -test showed in none of the subfields of the hippocampal formation a significant difference in neuronal number between young and aged rhesus monkeys (Table 4).

#### 4. Discussion

The present study shows that aging in rhesus monkeys is not associated with a (region-specific) neuron loss in any subfield of the hippocampal formation, and confirms and extends an earlier report by West et al. [65]. The latter study was performed with monkeys that had been behaviorally

tested. Three of five aged monkeys showed memory impairments similar to such memory decline after damage of the medial temporal lobe. However, the neuron numbers did not correlate with the impaired memory performance [65]. Since the neuronal loss cannot account for decreased cognitive abilities, changes in the blood supply to the brain may do so. In a previous study with rhesus monkeys from the same colony as in the present study, we showed that aging is associated with an increase in aberrations of capillary walls in the CA1 and CA3 regions of the hippocampus [25]. These microvascular aberrations had previously been shown in rats to correlate with cognitive impairments [11], and may deteriorate the passage of oxygen and nutrients to the neuropil, eventually leading to decreased memory performance in rhesus monkeys.

Rhesus monkeys in controlled laboratory conditions grow up under more or less constant conditions. West et al. [65] investigated the hippocampi of rhesus monkeys from another laboratory colony, i.e. with different genetic backgrounds. Minor individual differences in housing, such as feeding or the social position within the animal colony, may increase individual differences between rhesus monkeys not only from different colonies, but even from a single colony. In human studies, it is even likelier that larger interindividual differences in genetic background and life experience are a source of deviating results. The hypothalamic–pituitary–adrenal axis, for example, is subjected to complex interactions of the internal and external environment. Some individuals may have low glucocorticoid levels throughout their lives, whereas others show increased levels in their later lives [34]. Furthermore, stressful experiences may additionally modulate glucocorticoid levels [33], and aged individuals may be more vulnerable to such external influences than younger individuals. High levels of glucocorticoids have been shown to correlate with impaired hippocampal-dependent cognitive function in rodents [3], tree shrews [41,42], and humans [30,40].

Elevated glucocorticoid levels, whether from long-term glucocorticoid administration or prolonged stress, caused dendritic atrophy in the CA3 region in rats [60,71] and tree shrews [31], and may potentiate excitotoxic damage to hippocampal neurons [33]. However, by stereological analyses, long-term psychosocial stress in tree shrews did not affect the numbers of neurons in the hippocampus [57], nor were hippocampal neuron numbers influenced by chronic, stress-like levels of exogenous glucocorticoids in aged macaques [27]. Unfortunately, no individual data on cortisol levels or cognitive state exist for the human subjects, whose hippocampi had been stereologically investigated [55,63]. Still, it remains to be solved why aging in humans, and not in rhesus monkeys, is accompanied by region-specific hippocampal neuron loss. Concerning the neuron numbers in the CA1 region, however, it is most likely that no neuron loss occurs in normal aging humans. In fact, Simic et al. [55] might have included early undiagnosed cases of Alzheimer's disease in the study on the effects of normal human aging [47]. Neurons are lost from the entorhinal cortex already in very mild stages of Alzheimer's disease [16,49]. The latter study also shows a reduction of CA1 neurons in very mild and severe Alzheimer's disease, but not in nondemented healthy subjects of 60–89 years of age and in preclinical Alzheimer's disease cases [49]. Furthermore, the neuron loss in CA1 seemed to uniquely predict the duration and severity of Alzheimer's disease [8]. Interestingly, the study of Simic et al. showed a neuron loss in CA1 during normal human aging, and in the dentate gyrus specifically for Alzheimer's disease [55], whereas West only demonstrated a loss of CA1 neurons in Alzheimer's disease, but not in normal human aging [66].

The absence of hippocampal neuronal loss in rhesus monkeys does not seem to account for age-related impairment of cognitive functions. The main input to the hippocampal formation comes from layers II and III of the entorhinal cortex, such that neurons in layer II project to the outer molecular layer of the dentate gyrus and CA3, whereas layer III of the entorhinal cortex has additional direct connections to CA1 and subiculum [2,53,69]. With stereological techniques, several studies provided evidence that neurons in layer II of the entorhinal cortex do not disappear in the aging rhesus monkey [14,35,46,65], or in other entorhinal cortical layers of the nonhuman primate [35,46]. Also in cognitively normal human subjects, stereological analyses showed that neuron numbers in entorhinal cortical layers II, III, IV, or V/VI remained constant between 60 and 90 years of age [16,49].

Taken together, stereological studies so far were not able to demonstrate a loss of neurons in any subfield of the hippocampal formation or entorhinal cortex of aging nonhuman primates. Likewise, in humans, based on unbiased counting techniques, there is no evidence that neurons from any layer in the entorhinal cortex disappear with advanced age. However, as described above, studies on neuronal number in the hippocampal formation during normal human aging demonstrate a region-specific neuronal loss.

Except for the hippocampal formation, which plays a crucial role in cognitive processes like learning and memory [13], and the entorhinal cortex, which is the gateway to the hippocampal formation [69,70], other cortical areas in primates seem to be spared during aging, as well [46]. A recent stereological study dealing with the effect of aging in the primary visual cortex of rhesus monkeys showed no loss of large projecting neurons [22]. As far as nonhuman primates are concerned, stereological analyses of various brain structures strongly indicate that neuron numbers do not decline with age. Although the primary visual cortex of aged rhesus monkeys showed no electron microscopical sign of neuron loss, various types of neuroglial cells and pericytes were affected by age [45]. Also, in nonhuman primates, a decrease in white matter volume, as measured with magnetic resonance imaging, was correlated with an overall cognitive impairment [47]. In aging rhesus monkeys, changes in myelin occurs not only in the primary visual cortex, but also in the frontal cortex and the corpus callosum [48]. These alterations in myelin sheaths are supposed to be ubiquitous, and were shown to correlate with cognitive impairment.

Impairments in conduction velocity, due to changed characteristics of myelin, maybe in conjunction with changes in the cerebral blood flow [12] or supply [25], as indicated in nonhuman primates [25], may compromise neuronal cells in their functioning. Brain volume, cerebral metabolites and receptor activation can be captured in magnetic resonance spectroscopy and positron emission tomography [20], respectively, where specific information can be obtained about neurons, glial cells, energy metabolites, cell membranes, or second messenger systems. These *in vivo* methods also raise the possibility to perform longitudinal studies on aging-related changes in different compartments of the brain and may increase our insight in subtle changes in the aging process. At least for these underlying changes that eventually cause region-specific neuronal loss in the human hippocampus, the rhesus monkey may be an adequate animal model for normal human aging.

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